Effect and mechanism of a steroid alkaloid derivative on thymic stromal lymphopoietin production in mouse keratinocytes

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(ステロイドアルカロイド誘導体による thymic stromal lymphopoietin 産生の誘導作用とその作用メカニズムの解析)

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Background: Recently, treatment for autoimmune diseases has been revolutionized with the discovery that regulatory T cells (Tregs) have critical suppressive functions for maintenance of self-tolerance and prevention of immune responses. Thymic stromal lymphopoietin (TSLP), traditionally recognized as a potent promoter of T helper type 2 (Th2) cell-associated cytokine responses, also plays a pivotal role in augmenting Tregs development or conversion. Importantly, *in vivo* studies have confirmed that exogenous TSLP or TSLP-conditioned bone marrow DCs induces a tolerogenic immune response in type 1 diabetic mice and protects against diabetes by increasing the number of Tregs. In particular, TSLP has a direct effect on promotion of Tregs development in thymus and periphery, which might overcome the difficulty of current clinical trial protocols by *in vitro*- or *ex vivo*-expanded Tregs. Hence, induction of TSLP is a promising step in the development of novel therapeutics for treatment of autoimmune-mediated diseases. However, most of TSLP-producing chemicals are pollutants or toxicants and so, they can't be used further as a candidate drug to prevent autoimmune diseases.

Objective: To find a potent TSLP-inducing compound and make the molecular mechanism involved clear, then based on this, to provide a promising candidate drug for the treatment of autoimmune diseases.

Methods: A murine keratinocyte cell line, KCMH-1, which can constitutively produce a large amount of TSLP, was utilized for high-throughput screening of 2169 compounds on TSLP production in my lab. Five cell lines including mouse keratinocytes PAM212 and KCMH-1, human keratinocytes HaCaT and A431, and human bronchial epithelial cell BEAS-2B were used to confirm the inducible effect of the focused compound on TSLP production. PAM212 cells were selected for the detailed study on the effect and molecular mechanism of the compound in TSLP production. Cell viability, TSLP protein levels in the cell culture supernatant, and TSLP mRNA expression of PAM212 cells were determined by thiazolyl blue tetrazolium bromide (MTT) assay, enzyme-linked immunosorbent assay (ELISA), and quantitative real-time polymerase chain reaction (qRT-PCR), respectively. Proteins expression of phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2, phosphorylated IκB kinase (p-IKK) α/β and IκB in total cell lysate, and p65 in nuclear and cytosol fractions were measured by western blotting. Luciferase activity was determined using a dual-luciferase reporter assay system, while gene knockdown was carried out by transfecting Stealth™ RNAi or siRNA into cells using Lipofectamine™ RNAiMAX. Filamentous actin (F-actin) expression was measured with a laser scanning confocal microscope.

Results and Discussion: High-throughput screening of 2169 compounds yielded a steroid alkaloid derivative, code no. 02F04, with the IUPAC name of (4S,6aR,8aS,8bR,9S,9aR,12S,16aS,17aS,17bS)-6a,8a,9,12-tetramethyl-1,3,4,5,6,6a,6b,7,8,8a,8b,9,9a,10,11,12,13,15,16a,17,17a,17b-docosahydronaphtho[2',1':4,5]indeno[1,2-f]pyrido[1,2-c][1,3]oxazepin-4-ol. As shown in Fig. 1, 02F04, with chemical formula of $C_{28}H_{45}NO_2$ and molecular weight of 427.67, has a unique steroidal skeletal structure which is different from other TSLP-inducing chemicals reported previously but similar to the endogenous ligands of a nuclear receptor of liver X receptor (LXR), such as 22 (R)-hydroxycholesterol (22R-HC). 02F04 concentration- and time-dependently induced mRNA expression and protein production of TSLP in mouse keratinocyte cell lines of PAM212 (Fig. 2) and KCMH-1 cells, but not in any tested human epithelial cells. The different responses to 02F04 in mouse and human epidermal cells might be contributable to the species specificity. In particular, the activity of 02F04 was selective to TSLP because 02F04 did not induce other cytokines expression in PAM212 cells. As an analogue of LXR endogenous ligand, 02F04 increased ATP-binding cassette transporter A1 (ABCA1) expression quickly by regulation of nuclear receptor of LXR (Fig. 3A,B). However, LXR antagonist did not inhibit 02F04-induced TSLP production and LXR agonists did not induce TSLP production, indicating that 02F04-induced TSLP production did not involve LXR (Fig. 3C). Besides, 02F04 did not activate other major nuclear receptors, such as retinoic acid receptor, retinoid X receptor, peroxisome proliferator-activated receptor and glucocorticoids receptor, and that activation of vitamin D receptor did not induce TSLP production in PAM212 cells, suggesting that 02F04 induced TSLP production via activating signaling molecules rather than nuclear receptors.

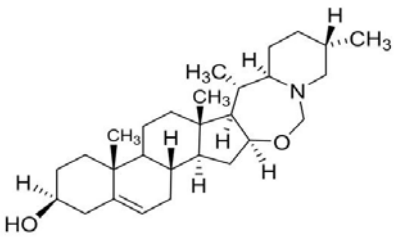

Fig.1 Chemical structure of 02F04

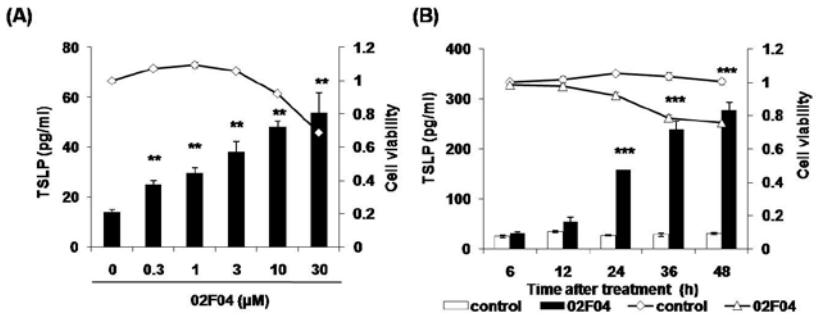

Fig.2 Induction of TSLP production by 02F04 in PAM212 cells

The upregulation of TSLP protein and mRNA levels by 02F04 treatment in PAM212 cells was significantly suppressed by inhibitors of pan-protein kinase C (PKC) (3 μM), Rho-associated protein kinase (ROCK), Gq/11, and ERK 1/2. Besides, compared with the

control group, 02F04 accelerated F-actin formation and induced a slow activation of p-ERK1/2. Moreover, small interfering RNA (siRNA)-mediated knockdown of Gq or G11 also suppressed 02F04-induced TSLP production. However, although IKK2 inhibitor suppressed 02F04-induced TSLP production, 02F04 only had a weak and not apparent effect on activating nuclear factor-κB (NF-κB) signal pathway. This was because NF-κB promoter activity induced by 02F04 from 1 to 10 μM was only increased 1.3- or 1.4-fold higher and besides, 02F04-induced change in IκB degradation, p-IKKα/β activation and nuclear translocation of p65 was too small to be detected. Taken together, it was suggested that 02F04-induced TSLP production was under the firm control of PKC, ROCK, Gq/11 and ERK1/2 signaling cascades, whereas the NF-κB signaling pathway has only a weak effect. In addition, I also found that 02F04-induced phosphorylation of ERK1/2 was suppressed by inhibitors of pan-PKC (3 μM), ROCK and Gq/11 accompanied by the decreased protein and mRNA levels of TSLP, indicating that PKC, ROCK and Gq/11 signaling cascades were the upstream of ERK1/2 signal pathway in 02F04-induced TSLP production. Furthermore, I demonstrated that Gq/11 was located in the upstream of ROCK signal cascade because the specific Gq/11 inhibitor of YM-254890 significantly reduced 02F04-induced actin stress fiber formation.

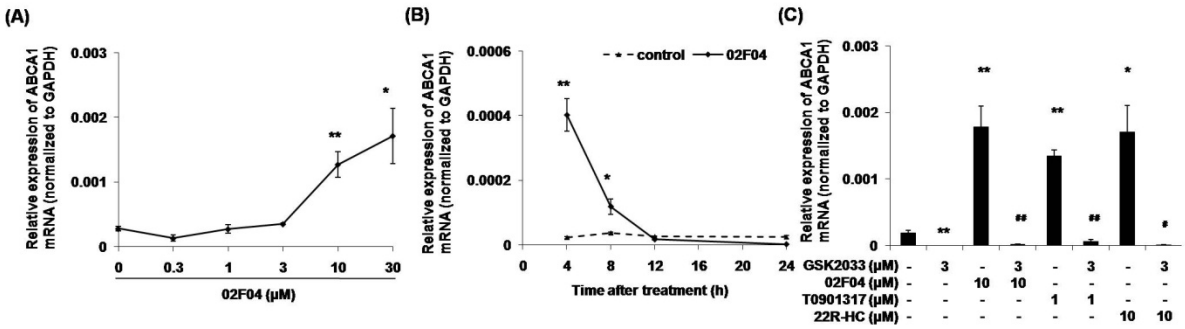

Fig.3 Involvement of LXR in 02F04-induced ABCA1 expression

Unexpectedly, though the inhibitors of phospholipase C (PLC), pan-PKC (10 μM) and PKCδ (3 and 10 μM) also significantly attenuated basal and 02F04-induced TSLP production, TSLP mRNA levels were not changed by PLC inhibitors, or increased by inhibitors of PKCδ at any tested concentrations and pan-PKC at 10 μM following an increase in p-ERK1/2 expression. These findings suggested that inhibition of PKCδ signaling pathway modulated basal but not 02F04-induced TSLP production in PAM212 cells by activating ERK1/2 signaling pathway. It was notable that pan-PKC inhibitor at 3 and 10 μM exhibited different effects on the expression of TSLP mRNA and phosphorylation of ERK1/2. This might be contributable to the low IC_{50} value of GF109203X. When the concentration was increased to 10 μM, GF109203X might exert other actions that are unrelated to and independent of PKC inhibition, so showing an opposite result. As to PLC signal pathway, it was involved in neither basal nor 02F04-induced TSLP production in PAM212 cells. However, PLC, PKC and PKCδ molecules played vital roles in the regulation of TSLP exocytosis from PAM212 cells, so treatment

with their inhibitors, especially at the high concentrations, resulted in the obvious reduction of TSLP protein level in the cell culture supernatants. Notably, it is unknown yet that whether ROCK, Gq/11 or ERK1/2 molecules can modulate the exocytosis of TSLP from PAM212 cells, which needs to be investigated with specific method in the future.

TSLP plays critical roles in modulating immune responses and maintaining tolerance to self-antigens by augmenting Tregs development or conversion. Besides, LXR also can modulate immune system function and homeostasis. 02F04 has been demonstrated to have the ability to induce production of TSLP and activation of LXR simultaneously, which might make it more beneficial in immunomodulation of autoimmune.

Conclusion: In a word, I found, for the first time, that 02F04, a steroid alkaloid as well as an analogue of the LXR endogenous ligand, induced TSLP production at the protein and mRNA levels in murine keratinocytes. In particular, the activity of 02F04 was selective to TSLP. Similar to LXR agonists, 02F04 rapidly increased ABCA1 expression through regulation of LXR. However, 02F04 induced a slow production of TSLP not by regulating nuclear receptors, but via activating distinct signal transduction pathways, including the firm control of PKC (not including PKCδ), ROCK, Gq/11, ERK1/2 and a weak effect on NF-κB signaling cascade. Besides, part of crosstalk mechanisms among the distinct signal transduction pathways were confirmed. That is, Gq/11, ROCK and PKC, as the upstream of ERK1/2 signaling pathway, and Gq/11, as the upstream of ROCK signaling pathway participated in 02F04-induced TSLP production. Moreover, PLC, PKC and PKCδ molecules also play important roles in exocytosis of TSLP from PAM212 cells (Fig. 4). My system has proven useful in identifying a novel TSLP-producing chemical. 02F04, with a unique skeletal structure compared with other TSLP-inducing chemicals previously reported, can increase ABCA1 expression rapidly by activating LXR and induce TSLP production slowly and continuously by triggering crosstalk mechanisms among the distinct signal transduction pathways. The dual roles of 02F04 make it a promising candidate drug for the treatment of autoimmune diseases.

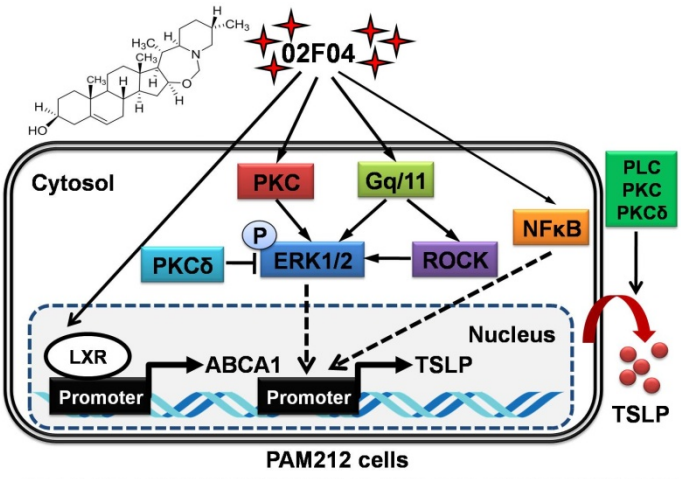

Fig.4 Putative mechanisms of 02F04 on TSLP production in PAM212 cells

別紙様式（Ａ４版）

論文審査結果の要旨

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論 文 題 目：Effect and mechanism of a steroid alkaloid derivative on thymic stromal lymphopoietin production in mouse keratinocytes
（ステロイドアルカロイド誘導体による thymic stromal lymphopoietin 産生の誘導作用とその作用メカニズムの解析）

本研究は、自己免疫疾患治療薬開発を目指し、制御性 T 細胞を誘導する活性を持つサイトカイン thymic stromal lymphopoietin (TSLP)の産生を誘導する化合物を探索し、そのヒット化合物の作用機構を解析したものである。

自己免疫疾患は、免疫応答が過剰となることが原因として生じる難治性疾患である。これまでその治療にはステロイド性抗炎症薬、免疫抑制薬、抗体医薬などが用いられているが、重篤な副作用が生じる場合があることなど問題点も多い。最近では、免疫抑制作用を持つ細胞を用いた治療法の開発も進んでいる。特に注目されているのが制御性 T 細胞の活用である。しかしこれまで、制御性 T 細胞の誘導を目的とした低分子化合物の開発はなされていない。そこで本研究では、最近制御性 T 細胞を誘導することが明らかにされたサイトカイン TSLP に注目し、その産生を選択的に誘導する化合物の探索を行った。マウスのケラチノサイト細胞株を用いたスクリーニングを行い、ステロイドアルカロイド骨格を持つ化合物（コード No. 02F04）を見出した。

02F04 はステロイド骨格を持つこと、TSLP の産生はいくつかの核内受容体により制御されていることが知られていたことから、まず種々核内受容体のアゴニスト、アンタゴニストを用いて核内受容体の寄与を解析した。その結果、02F04 は LXR を活性化する作用を持つものの、TSLP 産生誘導作用には核内受容体は関与していないことが示唆された。そこで次に、TSLP 産生ならびに分泌に関わるシグナル分子について解析した。02F04 による TSLP 産生は、これまで報告されている刺激剤と異なり、遅くかつ持続的である特徴があるが、ERK の活性化も非常に緩やかに生じること、ERK の活性化阻害薬は強く TSLP 産生を抑制することを示した。また、NF-κB の関与が小さいこと、PKCδが抑制的に作用すること、ROCK が TSLP の転写に寄与すること、PLC-PKC 経路が分泌に関わることなど、TSLP の産生機構として新たな知見を見出した。この研究成果は、02F04 の標的分子を探索する上で有用であり、さらに強力な TSLP 産生誘導化合物の発見に寄与するだけでなく、病理・生理的な TSLP の産生・放出制御機構の解明に大きく貢献するものである。

よって、本論文は博士（薬科学）の学位論文として合格と認める。